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In Vitro Microtubule-nucleating Activity of Spindle Pole Bodies in Fission Yeast *Schizosaccharomyces pombe*: Cell Cycle-dependent Activation in Xenopus Cell-free Extracts

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Abstract. The spindle pole body (SPB) is the equivalent of the centrosome in fission yeast. In vivo it nucleates microtubules (MTs) during mitosis, but, unlike animal centrosomes, does not act as a microtubule organizing center (MTOC) during interphase. We have studied the MT-nucleating activity of SPBs in vitro and have found that SPBs in permeabilized cells retain in vivo characteristics. SPBs in cells permeabilized during mitosis can nucleate MTs, and are recognized by two antibodies: anti-γ-tubulin and MPM-2 which recognizes phosphoepitopes. SPBs in cells permeabilized during interphase cannot nucleate MTs and are only recognized by anti-γ-tubulin. Interphase SPBs which cannot nucleate can be converted to a nucleation competent state by incubation in cytostatic factor (CSF)-arrested *Xenopus* egg extracts. After incubation, they are recognized by MPM-2, and can nucleate MTs. The conversion does not occur in *Xenopus* interphase extract, but occurs in *Xenopus* interphase extract driven into mitosis by preincubation with exogenous cyclin B. The conversion is ATP dependent and inhibited by protein kinase inhibitors and alkaline phosphatase. Purified, active, cdc2 kinase/cyclin B complex in itself is not effective for activation of MT nucleation, although some interphase SPBs are now stained with MPM-2. These results suggest that the ability of SPBs in vitro to nucleate MTs after exposure to CSF-arrested extracts is activated through a downstream pathway which is regulated by cdc2 kinase.

THE centrosome is the primary microtubule organizing center (MTOC)[1] in mammalian cells (reviewed by Mazia, 1984; Karsenti and Maro, 1986; Vandre and Borisy, 1989). During interphase it organizes the cytoplasmic network of microtubules (MTs) and during mitosis it gives rise to the spindle poles. Ultimately, through its organization of the MT network during interphase, the centrosome is involved in cell motility, the spatial organization of cytoplasm, ER, and Golgi formation and polarity of vesicle transport. The centrosome's function and its modification during the cell cycle and cell differentiation are important aspects of development.

The reorganization of cytoplasmic MTs into mitotic spindles at the onset of mitosis involves changes in the MT-nucleating activity of the centrosomes. In most animal cells, centrosomes consist of a pair of centrioles surrounded by pericentriolar material (PCM). The PCM, but not centrioles, has MT-nucleating activity (Gould and Borisy, 1977). The amount of PCM surrounding centrioles increases at the onset of mitosis (reviewed in Mazia, 1984; Leslie, 1990), and mitotic centrosomes in vitro are capable of nucleating more MTs than interphase centrosomes (Snyder and McIntosh, 1975; Kuriyama and Borisy, 1981). The MT-nucleating activity of centrosomes is also regulated during cell differentiation (reviewed in Karsenti and Maro, 1986). In epithelial cells in situ, for instance, centrosomes have almost no MT-nucleating activity during interphase (Zeligs and Wollman, 1979; Gorbsky and Borisy, 1985).

MT reorganization at the onset of mitosis involves changes in the dynamics of MTs nucleated from centrosomes. Fluorescence recovery after photobleaching (FRAP) of fluorescein-labeled tubulin injected into cultured mammalian cells (Saxton et al., 1984) and in vitro observations of MT dynamics at centrosomes using *Xenopus* cell-free extracts (Verde et al., 1990; Belmont et al., 1990) have shown that MT turnover during mitosis is more dynamic than during interphase. *Xenopus* cell-free extracts have been used to study cell cycle-dependent events as well as cell cycle regulation, because cytoplasmic extracts can be prepared that represent the biochemical states of mitosis or interphase. The conversion between mitotic and interphase extracts is possible in vitro by manipulating the activity of cdc2 kinase (Murray and Kirschner, 1989), a universal regulator of the cell cycle

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1. *Abbreviations used in this paper*: CSF, cytostatic factor; MT, microtubule; MTOC, microtubule organizing center; PCM, pericentriolar material; SPB, spindle pole body.

in eukaryotes (reviewed by Nurse, 1990). Verde et al. (1990) have shown that MT dynamics and steady state length can be influenced by cdc2 kinase in *Xenopus* cell-free extracts. The difference in MT dynamics in mitotic extracts is due to an increase in the frequency of transition from growing to shrinking MTs (Belmont et al., 1990). Gotoh et al. (1991) have shown that MAP kinase, which is activated during meiosis and mitosis in *Xenopus* eggs, can induce changes in MT dynamics in *Xenopus* cell-free extracts. However, no increase in MT-nucleating activity of centrosomes in mitotic extracts was observed in these studies. MT-severing activity has also been found in *Xenopus* mitotic extracts. This activity may contribute to disassembly of interphase MT networks at the onset of mitosis (Vale, 1991).

In fungi, the spindle pole body (SPB) is the equivalent of the mammalian centrosome. The SPB is an electron-dense plaque embedded in the nuclear envelope. In spite of the structural differences between mammalian centrosomes and fungal SPBs, they have some common components. One common component is γ-tubulin, a third member of the tubulin superfamily. It was first found in SPBs of *Aspergillus nidulans* (Oakley and Oakley, 1989; Oakley et al., 1990). γ-tubulin has recently been localized to the PCM of mammalian centrosomes (Zheng et al., 1991; Stearns et al., 1991), and to *Schizosaccharomyces pombe* SPBs (Horio et al., 1991). Other SPB components are MPM-2-reactive antigens, which were found in mammalian mitotic centrosomes (Vandre et al., 1984), *A. nidulans* mitotic SPBs (Engle et al., 1988), and *S. pombe* mitotic SPBs (Masuda et al., 1990).

We have chosen the fission yeast *S. pombe* as a model system for studying the regulation of MT-nucleating activity of centrosomes. In *S. pombe*, interphase SPBs do not nucleate MTs whereas mitotic SPBs do, thereby drastically reducing the complexity of the phenomenon we wish to study. Electron and immunofluorescence microscopy has revealed that during interphase in *S. pombe*, several cytoplasmic MTs run parallel to the long axis of the cell. The MTs do not appear to be nucleated from the SPB, however sometimes a MT seems to be laterally associated with the SPB. At the onset of mitosis, the cytoplasmic MTs disappear and the SPB then plays a prominent role in organizing the spindle within the nuclear envelope which does not break down (McCully and Robinow, 1971; Tanaka and Kanbe, 1986; Hagan and Hyams, 1988). This is in contrast to the budding yeast *Saccharomyces cerevisiae*, in which cytoplasmic MTs are nucleated from the cytoplasmic face of the SPB during G1 phase, and an intranuclear spindle-like structure is formed early in S phase which persists throughout the rest of the cell cycle (Byers and Goetsch, 1975). In *S. cerevisiae* SPBs have been isolated and several components identified (Rout and Kilmartin, 1990). In *S. pombe* the SPB has not been characterized at all biochemically. One regulatory gene for SPB duplication has been identified (Uzawa et al., 1990), and no components other than γ-tubulin and MPM-2 reactive antigens have been identified as a part of the SPBs.

We have developed an in vitro assay for studying MT nucleating activity of *S. pombe* SPBs. MPM-2 and antibodies against γ-tubulin were used as SPB markers. We demonstrate a mitosis-specific MT nucleation from SPBs in permeabilized cells. We show that CSF-arrested extracts from *Xenopus* unfertilized eggs can activate interphase SPBs so that they gain the ability to nucleate MTs. We also present evidence that the conversion in the extracts is under the control of cdc2 kinase. Based on these results we discuss potential mechanisms of SPB activation.

Materials and Methods

Preparation of Permeabilized Cells

Spheroplasts of nuc2.663 cells (Hirano et al., 1988) were prepared as described previously (Masuda et al., 1990). The nuc2 cells were grown in YPD medium (1% yeast extract, 2% polypeptone, 2% glucose) at 26°C, the permissive temperature. After the cell density reached 3–5 × 10^6/ml, cells were incubated at 36°C, the restrictive temperature, for 4–5 h. Cells were collected by low-speed centrifugation, washed once with 0.1 M Tris-H_2SO_4 (pH 9.4), and incubated in the same solution containing 10 mM DTT for 5 min at 36°C. They were then washed twice with YPD medium containing 1 M sorbitol (YPDS medium), resuspended at 10^8/ml in YPDS medium containing 0.6–1.0 mg/ml zymolyase 100T (Seikagaku Kogyo Co., Tokyo, Japan), and incubated at 36°C for 30–60 min. To prepare spheroplasts of wild-type ($972h^-$) cells, cells were grown at 26°C to a density of 2–4 × 10^6/ml. They were then collected and treated at 29°C as described above for nuc2 cells. To prepare spheroplasts of wild-type cells arrested during S phase, cells were grown at 26°C to the density of 1.5–3 × 10^6/ml and then incubated at 29°C for 4 h in the presence of 10 mM hydroxyurea. They were then treated at 29°C as described for nuc2 cells except that all solutions included 10 mM hydroxyurea.

Permeabilized cells were prepared as described previously (Masuda et al., 1990) with slight modifications. Spheroplasts were collected by centrifugation and washed three times with cold MESS solution (0.1 M MES, pH 6.5, 5 mM EDTA, 1 mM spermidine, 0.5 mM spermine, 1 M sorbitol, 20% DMSO, 0.1 mM rac-6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid (trolox; Fluka Chemical Co., Ronkonkoma, NY), 5 mM DTT, 0.2 mM PMSF, 1 μg/ml aprotinin, and other proteolytic inhibitors (Masuda et al., 1990). They were then resuspended in the same solution containing 0.5% Triton X-100 and incubated for 7 min on ice. Permeabilized cells were washed three times in MESS solution without sorbitol and then stored in the same solution in liquid N_2 for up to 3 mo. Frozen cells were thawed on ice, and washed briefly just before use with a solution containing 75 mM Pipes, pH 6.8, or Hepes, pH 7.4, 2.5 mM Mg-acetate, 5 mM EGTA, 40 mM β-glycerophosphate, 10% DMSO, 1 mg/ml ovalbumin, 3 mM GTP, 5 mM DTT, and proteolytic inhibitors.

Treatment of Permeabilized Cells with ATP or DNase I

Before permeabilized cells were processed for immunofluorescence with MPM-2 antibody, they were treated with ATP or DNase I to remove chromatin-associated antigens reactive with MPM-2 (Masuda et al., 1990). For ATP treatment, permeabilized cells were incubated for 10 min at room temperature in PMEG solution (75 mM Pipes, pH 6.8, 10 mM Mg-acetate, 5 mM EGTA, 40 mM β-glycerophosphate, 0.1 mM trolox, 5 mM DTT, and proteolytic inhibitors) containing 3 mM ATP, 3 mM AMPPNP, 20 μM taxol, and 20 μM detyrosinated neutrotubulin (bovine brain tubulin). In this solution, most *S. pombe* MTs were preserved, and spindle elongation was inhibited (Masuda et al., 1990). *S. pombe* MTs could be visualized by immunofluorescence with anti-tyrosinated α-tubulin (YL1/2), which recognized *S. pombe* tubulin, but not detyrosinated neurotubulin (Masuda et al., 1990). In some experiments, permeabilized cells were incubated for 10 min at room temperature in HMEG solution (the same as PMEG except 75 mM Hepes, pH 7.4, was substituted for Pipes) containing 6 mM ATP. In this solution, most MTs in permeabilized cells depolymerized. For DNase I treatment, permeabilized cells were incubated for 15–30 min on ice in a solution containing 50 mM Pipes, pH 7.0, 2.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 40 mM β-glycerophosphate, 0.1 mM trolox, 5 mM DTT, proteolytic inhibitors, 10% DMSO, and 30 U/ml DNase I (Boehringer-Mannheim Biochemicals, Indianapolis, IN).

Immunofluorescence Microscopy

Permeabilized cells were fixed with 2.6% paraformaldehyde and 0.2% glutaraldehyde in PMEG solution containing 10% DMSO without DTT and proteolytic inhibitors for 15 min. They were then extensively washed in antibody solution consisting of 0.1 M Pipes (pH 7.2), 1 mM $MgSO_4$, 1 mM EGTA, 1.83% L-lysine, 1% BSA, and 0.1% sodium azide and then incubated

for 30 min in the same solution. They were incubated at room temperature for 100–120 min with primary antibodies in antibody solution, followed by incubation with fluorescein or rhodamine-conjugated secondary antibodies.

Mouse mAb MPM-2 was kindly provided by Dr. Potu Rao (University of Texas M. D. Anderson Cancer Center, Houston, TX); and rat mAb against *S. cerevisiae* α-tubulin (clone YOL1/34) by Dr. John Kilmartin (Medical Research Council Laboratory of Molecular Biology, Cambridge, England); affinity-purified antibody against *A. nidulans* γ-tubulin by Dr. Berl Oakley (Ohio State University, Columbus, OH); affinity-purified antibody against *S. pombe* γ-tubulin by Dr. Tim Stearns (University of California, San Francisco, CA). The rat mAb YL1/2, which stains tyrosinated α-tubulin, was purchased from Accurate Chemical & Scientific Corp. (Westbury, NY), and affinity-purified secondary antibodies conjugated with fluorescein or rhodamine from Jackson Immuno Research Laboratory Inc. (Avondale, PA).

Preparation of Xenopus Cell-free Extracts

Extracts of *Xenopus laevis* unfertilized eggs arrested in metaphase of meiosis II by cytostatic factor (CSF-arrested extracts) were prepared as described (Murray and Kirschner, 1989). Interphase extracts were derived from CSF-arrested extracts by addition of 1/40 vol of 16 mM $CaCl_2$, 100 mM KCl, and 1 mM $MgCl_2$ and 1/50 vol of 5 mg/ml cycloheximide, with incubation for 30 min at 20°C, and then incubation on ice for 5 min in the presence of 1/40 vol of 16 mM EGTA (Belmont et al., 1990). Interphase extracts were induced to enter mitosis by incubation for 60 min at 23°C with 50 μg/ml cyclin Δ90, a nondegradable sea urchin cyclin B lacking the NH_2-terminal 90 amino acids (Murray et al., 1989). Cyclin Δ90 was kindly provided by Michael Glotzer (University of California, San Francisco, CA).

cdc2/GT-cyclin B complex was kindly provided by Dr. Mark Solomon (University of California, San Francisco, CA). It was a complex of cdc2 kinase and a recombinant cyclin fusion protein (GT-cyclin) consisting of a glutathione S-transferase moiety fused to an NH_2 terminally truncated cyclin B1 from sea urchin (Solomon et al., 1990), and with H1 kinase activity 5–10 times as high as CSF-arrested extracts in EB (80 mM β-glycerophosphate, pH 7.3, 15 mM $MgCl_2$, 20 mM EGTA) containing 5 mM glutathione, 1 mg/ml ovalbumin, 8% glycerol, 0.1 mM ATP, 1 mM DTT, and proteolytic inhibitors.

H1 kinase assays were performed by the method of Murray and Kirschner (1989) with slight modifications. H1 kinase activity of CSF-arrested extracts, interphase extract incubated with cyclin Δ90, and cdc2/GT-cyclin B diluted in EB or XB (used in experiments shown in Table I) was at least six times higher than that of interphase extracts.

MT Nucleation from SPBs

MTs in permeabilized cells were first depolymerized by incubation in HMEG solution containing 6 mM ATP at room temperature for 10 min. They were then incubated in PMEG solution containing 3 mM GTP, 20 μM taxol, and 20 μM neurotubulin (bovine brain tubulin) for 10 min at room temperature.

To study the effect of *Xenopus* cell-free extracts, permeabilized cells were incubated for 5-10 min in cell-free extracts containing 0.27 mg/ml creatine kinase, or in a control extract buffer (XB) (10 mM Hepes, pH 7.7, 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM EGTA, and 50 mM sucrose) containing 1/20 vol of energy mixture (150 mM creatine phosphate, 20 mM ATP, 2 mM EGTA, 20 mM $MgCl_2$, pH 7.7), 0.27 mg/ml creatine kinase, 5 mM DTT, and proteolytic inhibitors. In some control experiments, permeabilized cells were incubated in EB containing energy mixture, creatine kinase, DTT, and proteolytic inhibitors. After incubations, they were washed with cold XB/EB (7:3 mixture of XB and EB) containing 5 mM glutathione, 1 mg/ml ovalbumin, DTT, and proteolytic inhibitors. They were then incubated in PMEG solution containing GTP, tubulin, and taxol at room temperature.

To examine the effect of preformed MTs on SPBs, permeabilized cells were first treated with HMEG containing ATP, or incubated in CSF-arrested extracts followed by a wash in XB/EB and then incubated for 10 min at room temperature with MTs which were preformed by incubation of 20 μM tubulin and taxol in PMEG for 10 min and sheared by pipetting.

Immunoblot

Permeabilized nuc2 cells were treated in HMEG solution containing 6 mM ATP for 10 min, and washed briefly in the same solution. SDS sample buffer was then added to the cells, boiled for 3 min, and centrifuged. The supernatants were electrophoresed on SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. The membranes were stained with *Ponceau S*, and incubated for 1 h or more in blocking solution consisting of 2% FCS and 0.1% nonfat dry milk in 50 mM Tris, 1 mM EDTA, and 150 mM NaCl, pH 7.3. Primary antibodies (anti-*S. pombe* γ-tubulin diluted 1:1,000 in blocking solution, or MPM-2 diluted 1:500) were incubated with the blot for 2 h with rotary shaking at 200 rpm. Alkaline phosphatase-conjugated secondary antibodies were incubated with the blot for 1 h. Color development was performed by using the bromochloroindolyl phosphate/nitro blue tetrazolium substrate.

Table I. Effects of Xenopus cell-free Extracts and cdc2/GT-cyclin B Complex on MT-nucleating Activity of SPBs and SPB Staining with MPM-2

	MT nucleation	MPM-2 staining
	(%)	(%)
CSF-arrested extract, 10 min	79	93
interphase extract	26	15
interphase extract + Δ90 cyclin*	76	93
XB	0	4
EB	8	6
EB + cdc2/GT-cyclin B (X 1.5)‡	5	4
EB + cdc2/GT-cyclin B (X 2.5)‡	7	11
XB/EB	19	23
XB/EB + cdc2/GT-cyclin B (X1.5)‡	24	30
XB/EB + cdc2/GT-cyclin B (X1.5) 20 min‡	19	48
CSF-arrested extract		
+ 3 mM 6-dimethylaminopurine	10	6
+ 3 μM staurosporine	3	3
+ 31 U/ml alkaline phosphatase	0	0
+ 10 U/ml alkaline phosphatase	17	70
+ 15/ml apyrase	17	3
+ 10 μM colcemid	73	91
+ 250 μM vanadate	75	92

Permeabilized wild-type cells arrested at S phase with hydroxyurea were incubated for 10 min at room temperature in various kinds of *Xenopus* cell-free extracts, or in control buffer solutions including 1 mM ATP and ATP regenerating system with or without cdc2/GT-cyclin B. Cells incubated in *Xenopus* cell-free extracts were washed in XB/EB. They were then incubated in PMEG solution containing 20 μM neurotubulin and taxol for 10 min, and processed for immunofluorescence with YOL1/34 and MPM-2. N = 100–120.

* Before addition to permeabilized cells, interphase extract was incubated with 50 μg/ml Δ90 cyclin for 60 min.

‡ cdc2/GT-cyclin B in EB with H1 kinase activity 5× higher than CSF-arrested extracts was diluted 1.5× and 2.5× in EB, or 1.5× in XB (XB/EB as the final buffer).

Miscellaneous

Neurotubulin (bovine brain tubulin) and detyrosinated neurotubulin were isolated as described (Masuda et al., 1990). Staurosporine was kindly provided by Dr. Takashi Toda (Kyoto University, Kyoto, Japan). Taxol was kindly provided by Dr. Matthew Suffness at the National Cancer Institute. Alkaline phosphatase was purchased from Boehringer-Mannheim Biochemicals. Apyrase, AMPPNP and 6-dimethylaminopurine were purchased from Sigma Chemical Co. (St. Louis, MO).

Results

Finding a SPB Marker

Our ultimate goal was to demonstrate that an inactive interphase SPB could be converted to an active nucleating state in vitro using permeabilized *S. pombe* cells. Since the SPB is not visible using light microscopy, it was necessary to find a way to visualize it so that later, we could show that the SPB was indeed at the center of nucleated MTs. The visualization system we chose was indirect immunofluorescence since recently, potential SPB markers have become available. What

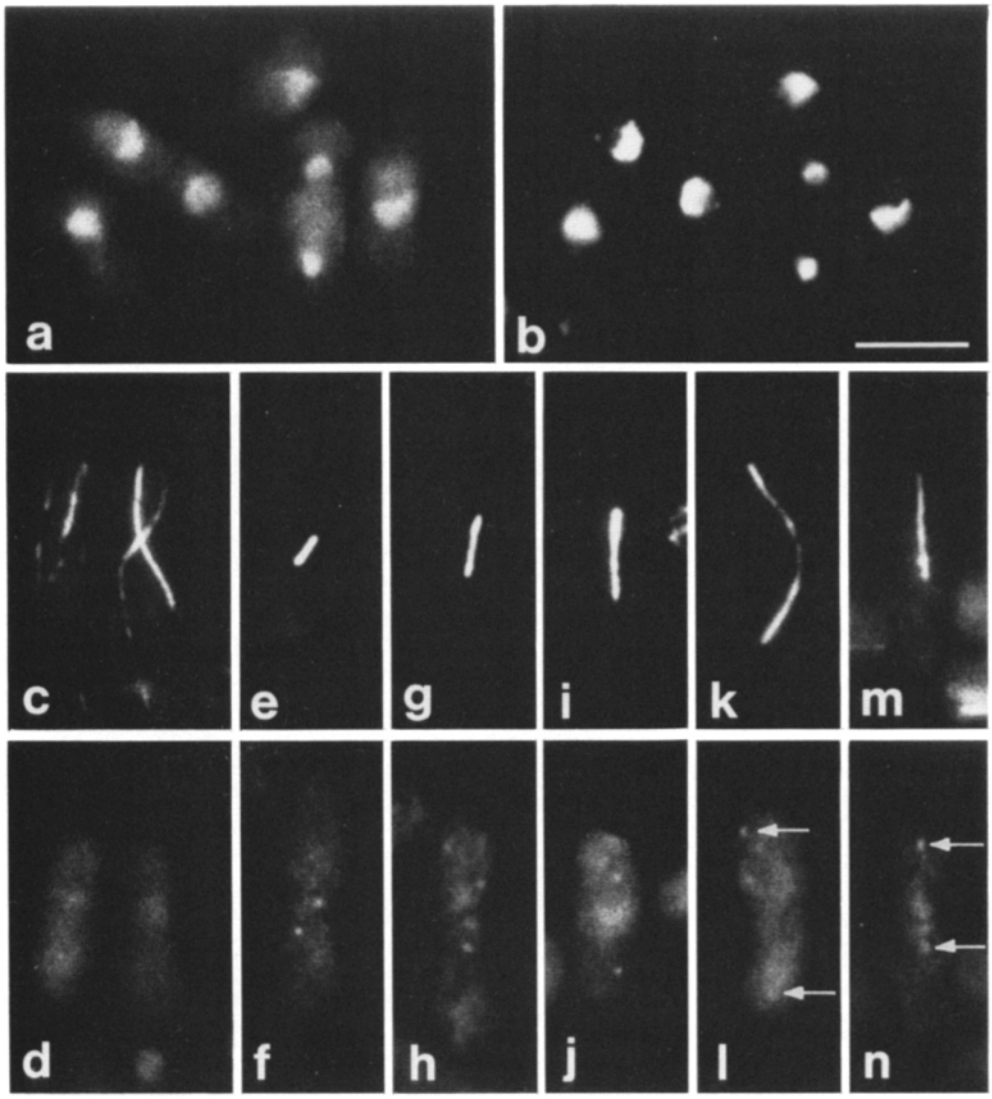

Figure 1. MPM-2 staining of mitotic SPBs. (*a–l*) Permeabilized wild-type cells grown asynchronously were processed for immunofluorescence before and after incubation in PMEG solution containing ATP, AMPPNP, taxol, and detyrosinated neurotubulin for 10 min at room temperature. (*a* and *b*) Interphase and mitotic cells before incubation; (*c–d*) interphase cells after incubation; (*e–l*) mitotic cells after incubation. Cells were double-stained with MPM-2 (*a*, *d*, *f*, *h*, *j*, and *l*) and DAPI (*b*) or anti-tyrosinated α-tubulin (YL1/2) that recognized *S. pombe* tubulin (*c*, *e*, *g*, *i*, and *k*). (*m* and *n*) Permeabilized wild-type cells were treated with DNase I in the presence of DMSO on ice, and processed for double-immunofluorescence with YL1/2 and MPM-2. (*m*) A mitotic cell stained with YL1/2; (*n*) stained with MPM-2. Arrows indicate the sites of SPBs stained with MPM-2. Bar, 5 μm.

was required was an antibody whose staining pattern was consistent with the location of the SPB as described by EM. (McCully and Robinow, 1971; Kanbe et al., 1990). In *S. pombe*, the unduplicated SPB is found to be embedded in the nuclear envelope during interphase. The SPB duplicates at the beginning of mitosis, and the separation of the SPBs is associated with spindle formation and elongation. During mitosis, the SPBs are located at the spindle poles.

One antibody that proved to be useful is MPM-2, a mitosis-specific mAb, that recognizes mitosis-specific phosphoepitopes (Davis et al., 1983). MPM-2 stains mammalian centrosomes in mitotic cells (Vandre et al., 1984) and mitotic SPBs in *A. nidulans* (Engle et al., 1988). We had also used MPM-2 to stain SPBs in permeabilized nuc2 cells, a temperature-sensitive mutant of *S. pombe* that arrests with a short spindle in a metaphase-like stage (Masuda et al., 1990). At that time, we had found that MPM-2 also stained *S. pombe* chromatin (Fig. 1, *a* and *b*) and that a wash with ATP removed enough chromatin-associated staining to reveal SPB staining with MPM-2.

To determine if MPM-2 could be used as a SPB marker in later nucleating experiments its staining pattern was characterized in cells from different stages of the cell cycle. An asynchronous mid-log phase population of wild-type cells was used as a source of cells in all stages of the cell cycle. Approximately 10% are mitotic cells with spindles of various lengths at different stages (Hagan and Hyams, 1988). They were permeabilized and treated with ATP under conditions that preserved the MTs in the cells (see Materials and Methods for detail), and processed for immunofluorescence with MPM-2, anti-tyrosinated α-tubulin (YL1/2) for staining *S. pombe* MTs, and DAPI for staining chromosomes. MPM-2 stained both ends of the spindles in mitotic cells from prophase to late anaphase (Fig. 1, *e–l*), but did not stain any structure in interphase cells that had cytoplasmic MTs running the lengths of the cells (Fig. 1, *c* and *d*). The intensity of SPB staining with MPM-2 in wild-type cells, however, was not as high as that in nuc2 cells. We also observed mitotic SPBs that were not stained with MPM-2, the percentage of which varied among experiments. Incubation of permeabilized cells with DNase I removed most chromatin-associated antigens that cross reacted with MPM-2 while leaving MPM-2 staining at the SPBs of mitotic nuc2 and mitotic wild-type cells (Fig. 1, *m* and *n*), but not in inter-

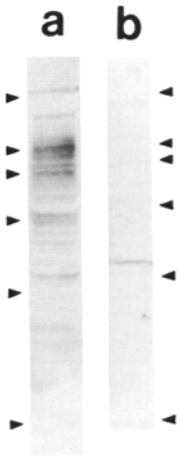

Figure 2. Immunoblots with anti-γ-tubulin and MPM-2 of proteins solubilized from permeabilized nuc2 cells. Permeabilized nuc2 cells arrested at the restrictive temperature were incubated in HMEG solution containing 6 mM ATP for 10 min. Proteins were then solubilized from the cells by boiling in SDS-sample buffer. (*a*) Immunoblot with MPM-2; (*b*) With anti-*S. pombe* γ-tubulin. Arrows indicate positions of molecular weight standards of 200, 116, 97, 66, 45, and 31 kD from the top to the bottom of the gels.

phase cells. An immunoblot of proteins solubilized from ATP-washed nuc2 cells shows that MPM-2 recognizes many proteins (Fig. 2 *a*). These results show that MPM-2 can be used as a mitotic SPB marker.

An antibody generated against *S. pombe* γ-tubulin (generously provided by Dr. Tim Stearns, University of California, San Francisco, CA) fulfilled the criteria of a SPB marker. γ-tubulin is a member of the tubulin superfamily. It is a component of *A. nidulans* SPBs (Oakley et al., 1990), of *S. pombe* SPBs (Horio et al., 1991), and of *Xenopus*, *Drosophila*, and mammalian centrosomes (Zheng et al., 1991; Stearns et al., 1991). Fig. 3 shows that this antibody recognizes SPBs in mitotic or interphase cells. γ-tubulin was recognized at the spindle poles in nuc2 cells arrested at the restrictive temperature (Fig. 3, *a* and *b*), or in wild-type mitotic cells (data not shown). γ-tubulin was recognized as a dot near the nucleus in interphase cells from an asynchronous culture (Fig. 3, *c–e*) or from a culture arrested in S phase with hydroxyurea (Novik and Mitchison, 1990) (Fig. 3, *f–k*). An immunoblot of proteins solubilized from ATP-washed nuc2 cells shows that the anti-γ-tubulin antibody principally recognizes a band of 48 kD. The molecular weight is comparable to the sizes of the proteins recognized by anti-γ-tubulin antibodies in crude extracts of mammalian, *Xenopus*, *A. nidulans*, and *S. pombe* cells (Oakley et al., 1990; Zheng et al., 1991; Stearns et al., 1991; Horio et al., 1991).

An antibody against *A. nidulans* γ-tubulin (generously provided by Dr. Berl Oakley, Ohio State University) stained nuc2 SPBs when the spindles were partially disassembled during permeabilization, but did not stain SPBs of complete spindles or any structure in interphase nuc2 cells (data not shown). However, after a wash with ATP the antibody clearly stained two spots in mitotic nuc2 and wild-type cells and one spot in interphase wild-type cells (Fig. 4).

Cell Cycle-specific Nucleation of MTs from the SPB In Vitro

It has been demonstrated using EM (Tanaka and Kanbe, 1986) and immunofluorescence with anti-tubulin (Hagan and Hyams, 1988) that in vivo, SPBs are the MTOC only during mitosis. Since the assay for the regulation of nucleation that we were developing was based on the idea that only mitotic SPBs are capable of nucleation, it was important to compare the nucleating ability of SPBs in vivo and in vitro.

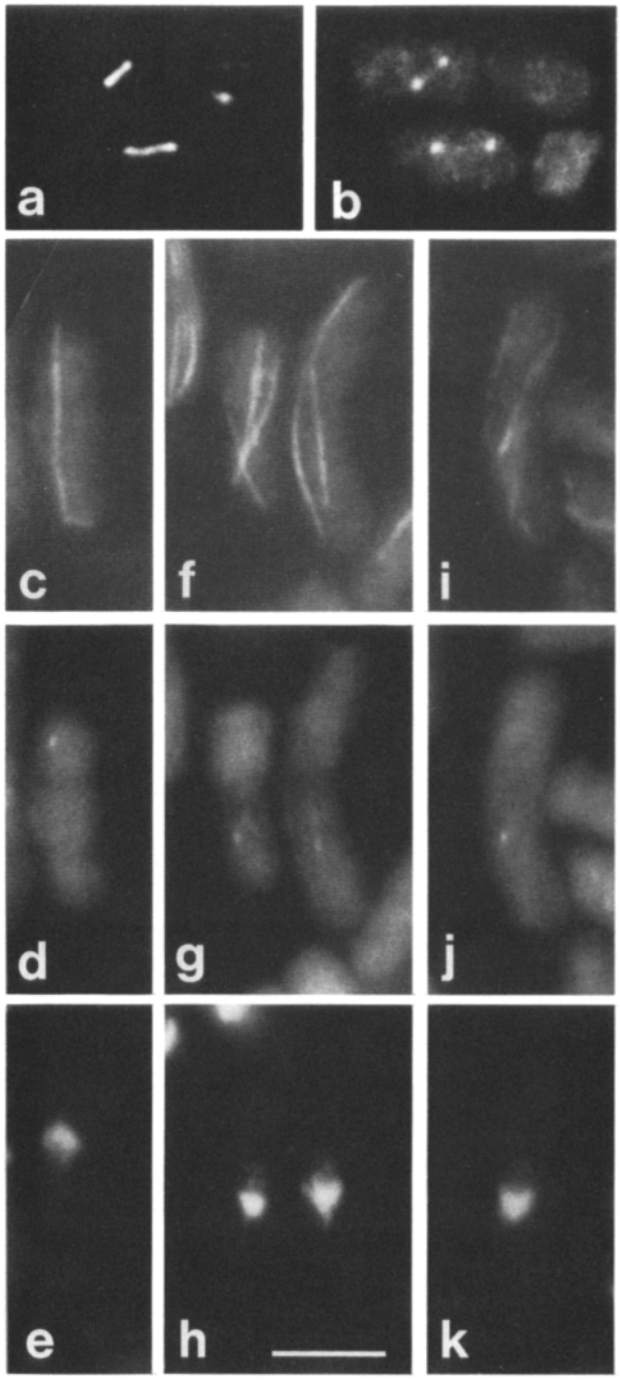

Figure 3. Anti-γ-tubulin staining of SPBs. (*a* and *b*) Permeabilized nuc2 cells arrested at the restrictive temperature were processed for double-immunofluorescence with YL1/2 (*a*) and anti-*S. pombe* γ-tubulin. (*c–k*) Permeabilized wild-type cells grown asynchronously (*c–e*) or arrested with hydroxyurea (*f–k*) were processed for triple-immunofluorescence with YOL1/34 (*c*, *f*, and *i*), anti-*S. pombe* γ-tubulin (*d*, *g*, and *j*), and DAPI (*e*, *h*, and *k*). Bar, 5 μm.

Three types of cells (nuc2, asynchronous wild-type, and hydroxyurea-arrested wild-type) were tested to avoid cell type-specific artifacts. All cells were permeabilized under conditions which preserved MTs. The cells representative of the in vivo situation were fixed without further manipulations, and triple-stained with anti-tubulin (YOL1/34), anti-γ-

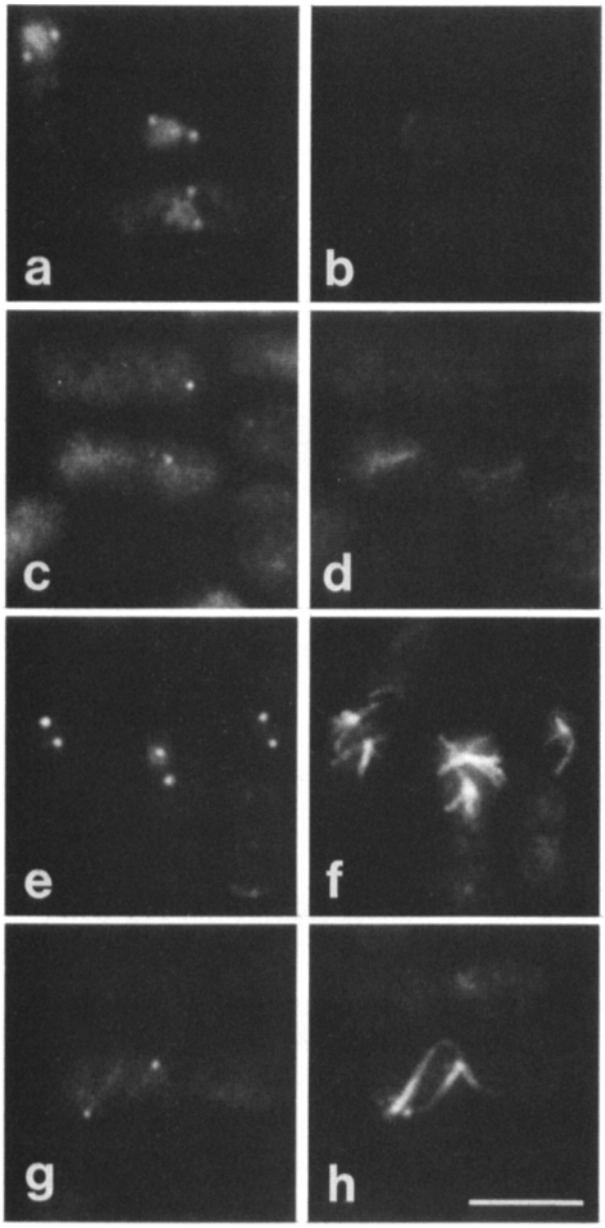

Figure 4. MT nucleation from mitotic SPBs. Permeabilized nuc2 cells arrested at the restrictive temperature and permeabilized wild-type cells grown asynchronously were incubated in HMEG solution containing ATP for depolymerizing MTs for 10 min. They were then incubated in PMEG solution containing 20 μM taxol and 20 μM tubulin (for wild-type) or 5 μM tubulin (for nuc2) for 10 min, and processed for double-immunofluorescence with anti-α-tubulin (YOL1/34) and anti-*A. nidulans* γ-tubulin, or MPM-2. (*a* and *b*) Mitotic nuc2 cells before addition of tubulin; (*c–d*) interphase wild-type cells after addition of tubulin; (*e* and *f*) mitotic nuc2 cells after addition of tubulin; and (*g* and *h*) a mitotic wild-type cell after addition of tubulin. (*a* and *e*) Stained with MPM-2; (*c* and *g*) with anti-*A. nidulans* γ-tubulin; and (*b*, *d*, *f*, and *h*) with YOL1/34. Bar, 5 μm.

tubulin, and DAPI. The cells which were used to characterize the in vitro nucleating ability of the SPBs were incubated in HMEG containing GTP and ATP at room temperature to depolymerize existing MTs (Fig. 4, *a* and *b*) and then challenged to nucleate at room temperature in PMEG containing 5 to 20 μM neurotubulin (bovine brain tubulin), 10 to 20 μM taxol, and 3 mM GTP before fixation and subsequent staining.

If nuc2 cells were used which were permeabilized after arrest at the restrictive temperature in vivo, >50% of the cells were mitotic with a short spindle, and the rest were in interphase (Masuda et al., 1990). In vitro, most of the SPBs in mitotic cells nucleated MTs (Fig. 4, *e* and *f*), but the SPBs in interphase cells did not. If wild-type cells were growing asynchronously when permeabilized, in vivo 10 to 12% of the cells were mitotic with a spindle and the rest were in interphase. 85 to 90% of the interphase cells had one MT from the cytoplasmic array that seemed laterally associated with the SPB (Fig. 2, *c–e*) and 5 to 10% of the interphase cells seemed to have one MT from the cytoplasmic array that had an end associated with the SPB. 3 to 5% showed no MT staining. In vitro, 85 to 90% of the cells had SPBs which did not nucleate MTs. Most of them had a single SPB per cell (Fig. 4, *c* and *d*), showing that they were in interphase. 10 to 15% of the cells had SPBs which nucleated MTs. Most of the cells which contained nucleating SPBs had two SPBs per cell (Fig. 4, *g* and *h*) showing that these cells were mitotic. If wild-type cells were synchronized by hydroxyurea arrest, in vivo 20 to 25% of the cells contained a cytoplasmic MT whose end appeared to be associated with the SPB (Fig. 2, *i–k*) and the rest contained a cytoplasmic MT which seemed laterally associated with the SPB (Fig. 2, *f–h*). In vitro, 15 to 25% of the cells contained a single SPB that nucleated.

Mitotic SPBs in nuc2 cells had the greater ability to nucleate MTs compared to mitotic SPBs in wild-type cells, since nuc2 SPBs nucleated MTs at lower concentrations of tubulin. Increasing the concentration of tubulin resulted in an increase in the percentage of nucleating mitotic SPBs and also resulted in an increase in the number and length of MTs. When 20 μM tubulin was added at 37°C in PMEG, most of mitotic SPBs nucleated MTs in the presence of 2 μM taxol. Decreasing the concentration of taxol lower than 2 μM resulted in lower fractions of SPBs which nucleated and lower numbers of MTs nucleated. If taxol was omitted, only a few percent of mitotic SPBs nucleated one or two MTs. In PME (PMEG without β-glycerophosphate), the nucleating activity of mitotic SPBs was greatly reduced, suggesting that maintaining the phosphorylated state of SPB proteins is important for MT nucleation.

It could be argued that the MTs that we saw around the SPB did not nucleate at the SPB but rather nucleated in solution, and were captured by the SPB. It has been shown that kinetochores of isolated chromosomes capture MTs when preformed MTs are added (Mitchison and Kirschner, 1985). To eliminate this possibility, tubulin and taxol were incubated under conditions which resulted in polymerization. Cells were added to the solution but the SPBs did not capture the preformed MTs (data not shown).

Interphase SPBs Preincubated in CSF-arrested Extracts Nucleate MTs

We sought to develop an in vitro system that could convert SPBs in interphase cells to a nucleation-competent state. CSF-arrested extracts from *X. laevis* eggs are known to cause nuclear envelope breakdown, chromosome condensation, and spindle formation in sperm nuclei and *Xenopus* nuclei

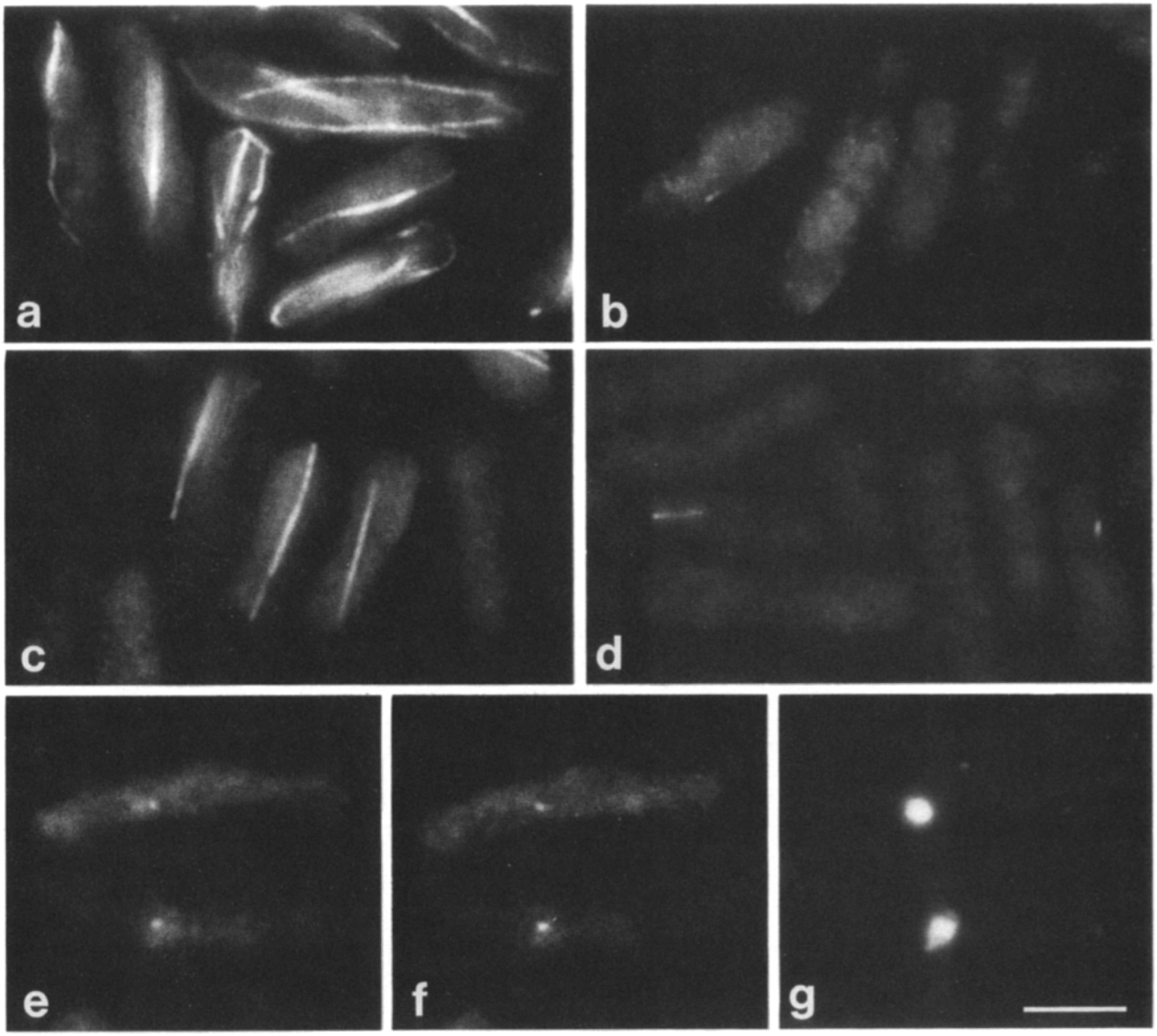

Figure 5. Effects of *Xenopus* cell-free extracts on interphase SPBs. Permeabilized wild-type cells arrested in S phase with hydroxyurea were incubated in *Xenopus* CSF-arrested or interphase extracts for 10 min at room temperature. (*a*) Cells before incubation; (*b, e–g*) after incubation in CSF-arrested extracts; (*c*) after incubation in interphase extract; and (*d*) after incubation in interphase extract followed by wash in XB/EB. (*a–d*) YOL1/34 staining; (*e–g*) triple staining with MPM-2 (*e*), anti-*S. pombe* γ-tubulin (*f*), and DAPI (*G*). Bar, 5 μm.

(Lohka and Maller, 1985; Murray and Kirschner, 1989). It has been shown that the onset of mitosis is regulated by a mechanism common to all eukaryote cells including *S. pombe* and *X. laevis* (reviewed by Nurse, 1990). Therefore we examined the effects of CSF-arrested extracts on SPBs in interphase permeabilized cells.

When permeabilized wild-type cells arrested at S phase with hydroxyurea were incubated in CSF-arrested extract, most cytoplasmic arrays of MTs disassembled during the first 5 min of incubation. After 10 min few MTs were left in the cytoplasm and no MTs were nucleated by the SPBs (Fig. 5, *a* and *b*). The time course of MT disassembly was similar to that in a buffer control (XB). Similar results were obtained with wild-type cells grown asynchronously. In contrast, when mammalian centrosomes are incubated in CSF-arrested extracts, MTs derived from the soluble *Xenopus* tubulin pool in the extracts are observed to radiate from the centrosomes (Verde et al., 1990; Belmont et al., 1990). After 10–30-min incubation in the CSF-arrested extracts, most SPBs stained with both anti-γ-tubulin and MPM-2 (Fig. 5, *e–g*). SPBs incubated for 30 min in CSF-arrested extracts stained more strongly by MPM-2 than those incubated for 10 min.

We examined if interphase SPBs preincubated in CSF-arrested extracts could nucleate MTs in the presence of brain tubulin and taxol. Permeabilized wild-type cells arrested by hydroxyurea in S phase were incubated in CSF-arrested extracts for 0–20 min. After the extract was removed, the cells were washed with buffer (XB/EB), and then incubated with tubulin and taxol in PMEG. Although some cytoplasmic MTs remained after incubation in the extract for less than 5 min, most of these MTs disassembled during the subsequent wash step. We found that >75% of SPBs in cells incubated for 5 or 10 min in CSF-arrested extract subsequently nucleated MTs in the presence of tubulin and taxol (Fig. 6,

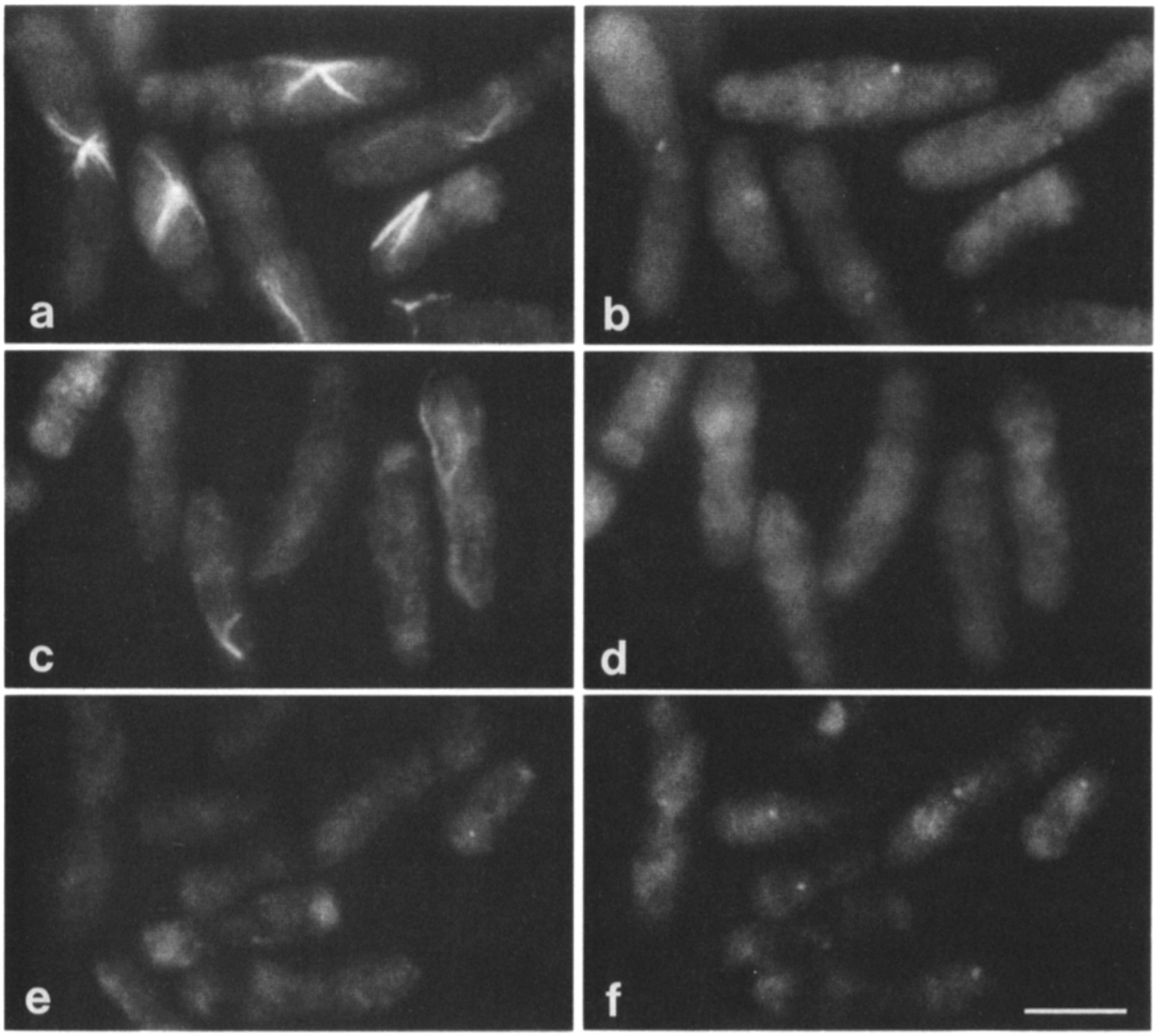

Figure 6. Effects of *Xenopus* cell-free extracts and cdc2 kinae/GT-cyclin B complex on MT nucleating activity of interphase SPBs. Permeabilized wild-type cells arrested with hydroxyurea were incubated in *Xenopus* CSF-arrested (*a* and *b*) or interphase (*c*, *d*) extract for 10 min at room temperature (*e* and *f*). They were washed in XB/EB and incubated in 20 μM tubulin and taxol for 10 min. (*a*, *c*, and *e*), YOL1/34 staining; and (*b*, *d*, and *f*), MPM-2 staining. Bar, 5 μm.

a and *b*; Table I). Both shorter and longer incubations resulted in a lower fraction of SPBs capable of nucleating MTs and in lower number of MTs nucleated from SPBs. If SPBs were incubated in tubulin and taxol in the absence of β-glycerophosphate, no MTs were nucleated at the SPBs, suggesting that maintaining the phosphorylated state of SPB proteins is important for MT nucleation. Similar activation of MT nucleation from interphase SPBs was observed when the same experiment was repeated using asynchronous wild-type cells.

It may be possible that very short MT seeds, not detectable by immunofluorescence, could be formed on SPBs in CSF-arrested extracts, and added tubulin could polymerize on the seeds. To exclude this possibility, the effect of colcemid on SPB activation was examined. Vale (1991) has shown that 5 μM colcemid inhibits endogenous tubulin assembly in *Xenopus* mitotic extracts. Addition of 10 μM colcemid to the mitotic extract had no effect on the conversion of interphase SPBs to mitotic SPBs (Table I), suggesting that SPBs are modified in CSF-arrested extracts by a mechanism other than *Xenopus* tubulin addition.

The Activation of MT Nucleation from SPBs Is under the Control of cdc2 Kinase in Xenopus Egg Extracts

We examined the effect of interphase egg extracts on interphase SPBs. Mitotic extracts from CSF-arrested eggs can be converted to an interphase state in vitro by adding calcium to inactivate CSF and allow cyclin degradation (Murray et al., 1989). Conversely, interphase extracts can be converted to a mitotic state by adding exogenous cyclin (Murray et al., 1989; Solomon et al., 1990). These processes do not require protein synthesis, since they occur in the presence of cycloheximide. When permeabilized wild-type cells arrested with

hydroxyurea were incubated with the interphase extract, cytoplasmic MTs disassembled more slowly than in mitotic extract (Fig. 5 *c*). After 10 min, one or two cytoplasmic MTs still remained in many of the permeabilized cells. However, most of the remaining MTs depolymerized when the interphase extract was removed and the cells washed with XB/EB (Fig. 5 *d*). These permeabilized cells were then incubated in the presence of tubulin and taxol and processed for double-immunofluorescence with anti-tubulin and MPM-2 to see whether the SPBs nucleated MTs and were recognized by MPM-2 (Fig. 6, *c* and *d*; Table I). Only 15 to 30% of SPBs were observed to nucleate MTs. Approximately 20–30% of cells showed no MT nucleation from SPBs, but had one or two cytoplasmic MTs. The remaining cells had no MTs. About 5 to 15% of the SPBs stained with MPM-2, and most of these also had nucleated MTs.

Interphase extract was converted to a mitotic state by incubation with cyclin Δ90 (generously provided by Michael Glotzer, University of California, San Francisco, CA), that is a nondegradable form of sea urchin cyclin B1 (Murray et al., 1989). After the treatment, the extract effectively converted interphase SPBs into mitotic ones (Table I). These results suggest that the activation of MT nucleation at SPBs in *Xenopus* egg extracts is a cell cycle–dependent event regulated by cdc2 kinase.

cdc2/GT-cyclin B1 Complex Does Not Directly Activate MT Nucleation from SPBs

We determined whether cdc2 kinase directly activated the MT-nucleation activity of SPBs. Solomon et al. (1990) have shown that a recombinant cyclin fusion protein (GT-cyclin B1) consisting of a glutathione S-transferase moiety fused to an NH_2 terminally truncated cyclin B1 from sea urchin activates histone H1 kinase when added to *Xenopus* egg interphase extract. The active cdc2/GT-cyclin B1 complex can be isolated from the interphase extract by affinity chromatography on glutathione Sepharose followed by elution with glutathione (Solomon et al., 1990; Pfaller et al., 1991). Permeabilized wild-type cells were incubated with cdc2/GT-cyclin B1 complex (generously provided by Dr. Mark Solomon, University of California, San Francisco, CA), and then incubated in the presence of tubulin and taxol. No increase in percentage of SPBs nucleating MTs was observed when compared to permeabilized cells incubated with control buffers (Table I). However, the percentage of SPBs that stained with MPM-2 increased after incubation with the cdc2/GT-cyclin B1 complex in XB/EB for 20 min (Table I; Fig. 6, *e* and *f*). These results suggest that incubation in the cdc2/cyclin B1 complex is not sufficient to activate MT nucleation from SPBs, but results in phosphorylation of some SPBs.

Activation of MT Nucleation Requires Protein Phosphorylation

To attempt to understand the relationship between nucleation and MPM-2 staining we investigated whether the conversion of interphase SPBs to mitotic SPBs in CSF arrested extract is dependent on protein phosphorylation. Permeabilized wild-type cells were incubated in CSF-arrested extract in the presence of enzymes or inhibitors added to the extract just before the incubation. They were then incubated in the presence of tubulin and taxol, and processed for immunofluorescence with anti-tubulin and MPM-2 (Table I). Addition of apyrase depletes the extracts of ATP and results in inhibition of SPB conversion. Both MT-nucleating activity and MPM-2 staining of the SPB were inhibited. Protein kinase inhibitors, 3 mM 6-dimethylaminopurine (Verde et al., 1990) or 3 μM staurosporine (Ruegg and Burgess, 1989) were effective in blocking conversion of the SPB. Alkaline phosphatase was also an effective inhibitor, although MT nucleating activity was more sensitive than staining of the SPB by MPM-2. These results suggest that SPBs acquire MPM-2 staining by protein phosphorylation, and that phosphorylation of proteins other than MPM-2 reactive antigens may be important for SPB activation.

Verde et al. (1991) have demonstrated taxol-induced MT aster formation in *Xenopus* CSF–arrested extracts. Aster formation requires both protein phosphorylation and cytoplasmic dynein, and is inhibited by addition of 50 μM vanadate. We studied whether similar factors were involved in the conversion of interphase SPBs to mitotic activity. Addition of 250 μM vanadate to CSF-arrested extracts had no effect on the conversion (Table I).

Discussion

We have developed an in vitro assay for studying the regulation of MT nucleation by SPBs in fission yeast. Using the assay, we demonstrated mitosis-specific MT nucleation from SPBs in vitro, and showed that CSF-arrested *Xenopus* extracts can convert SPBs which are incapable of nucleation to a nucleation-competent form. This conversion is under the control of cdc2 kinase. The regulation of SPB nucleation by CSF-arrested extracts from *Xenopus* is surprising since MTOCs in *S. pombe* and *Xenopus* are structurally dissimilar. SPBs in *S. pombe* are an electron-dense plaque embedded in the nuclear envelope, whereas centrosomes in *Xenopus* are composed of centrioles surrounded by amorphous PCM. Our results suggest that SPBs and centrosomes have common components required for MT nucleation, and that MT-nucleating activity of these evolutionary diverse structures is regulated by similar, cell cycle–dependent, mechanisms.

Components of SPBs and MT Nucleation

We have identified γ-tubulin as a component of SPBs using antibodies directed against them. γ-tubulin is localized at interphase and mitotic SPBs, and does not dissociate from the SPB under conditions which depolymerize spindle and cytoplasmic MTs in vitro. The γ-tubulin gene was identified as an extragenic suppressor of a β-tubulin mutant in *A. nidulans* (Oakley and Oakley, 1989). A null mutation results in a complete absence of the mitotic spindle and is lethal (Oakley et al., 1990). Immunolocalization with antibodies against γ-tubulin has shown that γ-tubulin is a component of *A. nidulans* SPBs (Oakley et al., 1990), *S. pombe* SPBs (Horio et al., 1991), and of *Xenopus* and mammalian centrosomes (Zheng et al., 1991; Stearns et al., 1991). γ-tubulin remains localized at centrosomes in cultured cells from *Xenopus* when cytoplasmic MTs are completely depolymerized by nocodazole (Stearns et al., 1991). These results suggest that γ-tubulin is a genuine component of SPBs and centrosomes.

Based on genetic and immunocytological results, Oakley et al. (1990) have proposed that γ-tubulin is tightly bound to

SPBs and interacts with β-tubulin to attach MTs to SPBs. They have also suggested that posttranslational modification of γ-tubulin may be a mechanism for cell cycle specific regulation of MT assembly. We have observed that staining intensity of SPBs with anti-γ-tubulin does not change much during the cell cycle, or after incubation in CSF-arrested extracts (data not shown). To study the role of γ-tubulin in MT nucleation, it must be determined if γ-tubulin is modified before SPB activation.

MPM-2 recognizes mitotic SPBs by immunofluorescence microscopy. MPM-2 has been shown to recognize mitotic mammalian centrosomes (Vandre et al., 1984), and *A. nidulans* mitotic SPBs (Engle et al., 1988). It is likely that MPM-2 recognizes phosphorylated components of SPBs, because MPM-2 reacts with mitosis-specific phosphorylated epitopes (Davis et al., 1983), because incubation with cdc2 kinase/GT-cyclin B1 complex increases the number of SPBs stained by MPM-2, and because ATP deletion, protein kinase inhibitors, or alkaline phosphatase inhibit acquisition of MPM-2 staining by SPBs incubated in CSF-arrested extracts.

Centonze and Borisy (1990) have shown that mammalian centrosomes lose MT-nucleating activity after incubation with MPM-2, suggesting that the phosphorylated epitope recognized by MPM-2 is important for MT nucleation. In contrast, our results suggest that the presence of MPM-2 reactive antigens may not be sufficient for MT nucleation by *S. pombe* SPBs. Although staining with MPM-2 is a good marker for mitotic SPBs which can nucleate MTs in vitro, there are several examples of SPBs that stain with MPM-2 that do not nucleate MTs. When incubated in CSF-arrested extract for >20 min, interphase SPBs that had acquired the ability to nucleate MTs lose it yet are strongly stained by MPM-2. After incubation with cdc2/GT-cyclin B1, some SPBs are recognized by MPM-2, but cannot nucleate MTs. Low concentrations of alkaline phosphatase inhibit activation of MT nucleation, but not acquisition of MPM-2 reactivity. We have also observed SPBs that do not stain with MPM-2 but which do nucleate MTs. After incubation in interphase extracts, ~15–30% of interphase SPBs nucleate MTs, and almost half of them do not stain with MPM-2. Since there is no consistent pattern in the available data, the most that can be said about the relationship between MPM-2 staining and nucleation is that they appear to be mitosis specific.

Mitosis-specific MT Nucleation

We have shown that in *S. pombe* only mitotic SPBs nucleate MTs in vitro. Our results are consistent with in vivo observations. Hagan and Hyams (1988) have observed by immunofluorescence with anti-tubulin, that during telophase, interphase arrays of cytoplasmic MTs are organized at the middle of the cell where the septum will be formed. During interphase, no aster-like cytoplasmic MT network is observed at the SPB, and cytoplasmic MTs run parallel to the long axis of the cell. We have observed that in permeabilized interphase cells, one to several cytoplasmic MTs run parallel to the long axis of cell, and one of them is always close to the SPB. These results suggest that SPBs are not a MTOC for the cytoplasmic array of MTs during interphase, although there may be some interaction between the cytoplasmic face of the SPB and the sides of cytoplasmic MTs.

In vivo, SPBs nucleate MTs from their nuclear and cytoplasmic faces at different stages of mitosis (Tanaka and Kanbe, 1986; Hagan and Hyams, 1988). From the beginning of mitosis through metaphase when cdc2 kinase is active, MTs nucleate only from the inner face of the SPB to form the spindle. Extranuclear MTs nucleate on the cytoplasmic face of the SPB as nuclear elongation starts. Nuclear elongation occurs at anaphase which is also the time at which cyclin is degraded and cdc2 kinase is inactivated. All MTs associated with the SPBs disappear by the end of telophase. Therefore, it seems that the ability of SPB to nucleate MTs is altered in conjunction with the activity of cdc2 kinase. We presume that more than a half of MTs nucleated from mitotic SPBs, or SPBs preincubated in CSF-arrested extracts, are intranuclear. The data which supports this idea is as follows. First, only intranuclear MT staining is seen in vivo when cdc2 kinase is active. Next, we have observed MT nucleation from both faces of the SPBs of nuc2 spindles in vitro by pulse labeling of spindles with biotin-labeled tubulin. More MTs seemed to nucleate on the nuclear face (Masuda et al., 1990). We have also observed that SPBs activated by incubation in CSF-arrested extracts nucleate more MTs toward the nucleus than toward the cytoplasm (H. Masuda, unpublished data). The MT nucleation at both faces of SPBs in vivo may be activated by the different mechanisms, or by the same mechanism, but what appears to be clear at this stage is that the pattern of nucleation is sensitive to the activity of cdc2 kinase. EM of SPBs nucleating MTs is necessary to investigate how both faces of the SPBs nucleate MTs in vitro.

We have observed that in 20–25% of wild-type cells arrested with hydroxyurea, one of the cytoplasmic MTs seems to terminate at the SPB in vivo as judged by immunofluorescence (Fig. 3), and similar percentages of SPBs nucleate in vitro (Table I). In contrast, similar termination of cytoplasmic MTs at the SPBs is observed in <10% of wild-type cells grown asynchronously. These results suggest that treatment with hydroxyurea results in the accumulation of some cells in a state in which only cytoplasmic MT nucleation is activated. The in vitro MT nucleation we have observed in cells treated with hydroxyurea may be due to this type of activation.

Mechanism for Activation of MT Nucleation from SPBs

We have shown that incubation in CSF-arrested extracts converts interphase SPBs to mitotic activity. Interphase SPBs are recognized by the mitotic SPB marker, MPM-2. The SPBs show no MT-nucleating activity in the extracts, but, like mitotic SPBs, nucleate MTs when brain tubulin and taxol are added, after the removal of the extract. The activation of nucleating ability is due to modification of SPBs by a mechanism other than addition of *Xenopus* tubulin to the SPB. This statement is supported by data which shows that addition of colcemid, an inhibitor of tubulin polymerization, to CSF-arrested extracts has no effect on the ability of the extracts to convert a nucleation incompetent SPB to a nucleation competent form.

Behavior of SPBs in *Xenopus* cell-free extracts is quite different from that of mammalian centrosomes. Mammalian centrosomes nucleate MTs in both CSF-arrested and interphase extracts (Verde et al., 1990; Belmont et al., 1990). In contrast, *S. pombe* SPBs nucleate no MTs in the extracts.

This difference suggests that non-yeast tubulin is not a good substrate for polymerization onto *S. pombe* SPBs in the absence of taxol. The fact that *S. pombe* SPBs do not nucleate MTs in CSF-arrested extracts allows us to study nucleation, and its regulation, in a system free of in vivo complexity. For example, Bre and Karsenti (1990) have shown that the number of MTs-nucleated from centrosomes is influenced by factors that affect MT dynamics such as MT-associated proteins. However, the in vitro assay we have developed will permit us to study the regulation of MT-nucleation independently of factors that influence MT dynamics.

The conversion of interphase SPBs to mitotic SPBs is under cell cycle control. CSF-arrested extracts contain active maturation promoting factor (MPF) consisting of cdc2 kinase bound to cyclin. We have shown that interphase SPBs can be converted to mitotic activity in CSF-arrested extract or in an interphase extract preincubated with exogenous cyclin B which drives it into mitosis, but not in interphase extract. These results suggest that SPB function is ultimately regulated by cdc2 kinase. The SPB activation seems likely to require the activity of a protein kinase, since it is ATP-dependent and is inhibited by protein kinase inhibitors and alkaline phosphatase. Alfa et al. (1990) have shown that anti-cdc2 and anti-cyclin B (cdc13 cyclin) recognize mitotic SPBs in *S. pombe*. Anti-cdc2 also recognizes mitotic centrosomes in mammalian cells (Riabowol et al., 1989; Bailly et al., 1989). These observations suggest the involvement of cdc2 kinase in activation of SPB or centrosome function at the onset of mitosis. However, purified cdc2 kinase/GT-cyclin B1 complex does not activate MT nucleation from SPBs. These results suggest that other factors besides cdc2 kinase are also required for the activation. Since cyclin Δ90, a truncated derivative of cyclin B1, can replace both cyclin A and B during the *Xenopus* cell cycle in vitro (Murray et al., 1989), it is unlikely that this factor is cdc2 kinase in a complex with a different cyclin other than cyclin B.

Our results can be explained by either of two models. In the first model, a downstream protein kinase activated by cdc2 kinase would phosphorylate a component of the SPB resulting in MT nucleation. In the second model, a factor that is required for MT nucleation would be recruited by the SPBs from the *Xenopus* extract. The factor could be *Xenopus* MT-nucleating material, or an activator of MT-nucleating material which is already associated with the SPB. The activity of the factor, or the recruitment of the factor would be regulated by cdc2 kinase. Although the latter model is more complicated, there are several examples in the literature that demonstrate the recruitment of cytoplasmic proteins to the centrosomes at the beginning of mitosis. The amount of PCM increases during mitosis compared to that during interphase (Rieder and Borisy, 1982), and mitotic centrosomes nucleate more MTs in vitro than interphase centrosomes (Snyder and McIntosh, 1975; Kuriyama and Borisy, 1981). Immunofluorescence studies using human autoimmune sera that recognize mitotic centrosomes (Sager et al., 1986; Leslie et al., 1991) show that proteins recognized by the sera accumulate at centrosomes at the onset of mitosis.

Xenopus eggs contain a stockpile of MT-nucleating material that is used for centrosome formation during early stages of development. It has been shown that CSF-arrested *Xenopus* extract has aster-forming activity in the presence of taxol (Verde et al., 1991). Aster formation in vitro requires phosphorylated factors and cytoplasmic dynein, and is inhibited by vanadate. In contrast, SPB activation is not inhibited by vanadate, suggesting that MT nucleation from activated SPBs is a process different from cytoaster formation in vitro. Although we do not favor either of the two models at this point, the fractionation of CSF-arrested extract and identification of the components required for SPB activation should clarify the mechanism of SPB activation.

In conclusion, we have developed an in vitro assay for studying the mechanism of *S. pombe* SPB activation. The ability of CSF-arrested extract to convert interphase SPBs to mitotic activity will enable us to isolate the factors responsible for the conversion. The factor could have a universal role in activating MTOCs during mitosis. Finally the application of this assay to appropriate cdc mutants will be useful in studying the role of the mutant protein in SPB activation.

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